

Amendments to the Specification:

Please replace the paragraph at page 23, from line 2 through line 4, with the following paragraph:

-- Figure 1 shows a sequence alignment of amino acid sequences of several RKIP family member proteins[.], including the following sequences: SEQ ID NO:2, Human; SEQ ID NO:3, Murine; SEQ ID NO:4, Drosophila; SEQ ID NO:5, C. Elegans; SEQ ID NO:6, Antirrhinum; SEQ ID NO:7, Aradopsis; SEQ ID NO:8, Yeast. The brackets above the alignment depict the RKIP motif, and the arrows indicate residues shown to be necessary for RKIP activity. --

Please replace the paragraph at page 23, from line 5 through line 12, with the following paragraph:

-- FIG. 2 shows FIGS. 2a-2f show in vitro interaction of RKIP with components of the ERK pathway. Fig. 2a) RKIP interacts with BXB, but not control baits in the yeast two hybrid system. Fig. 2b and Fig. 2c) Binding of recombinant BXB, full-length Raf-1, MEK-1 andERK-2, to GST-RKIP beads. "Input", 1% of the respective proteins used in binding reactions; "GST", GST-beads. Fig. 2d and Fig.2e[c])Co-immunoprecipitation of RAF-1, MEK and ERK with RKIP in Rat-1 cells. The RKIP antiserum does not precipitate recombinant Raf-1, MEK-1 and ERK-2 proteins individually. Fig. 2f[d])Co-localization of Raf-1 and RKIP in 208F fibroblasts by confocal microscopy. "Antigen competition", antisera were pre-absorbed with their cognate antigens.--

Please replace the paragraph at page 23, from line 13 through line 21, with the following paragraph:

-- FIG. 3 shows FIG. 3a – Fig. 3g show that inhibition of endogenous RKIP activates AP-1 dependent transcription. Fig. 3a – Fig. 3c) Microinjection of anti-RKIP antibodies. Quiescent Rat-1 cells were microinjected with the indicated reporter plasmids and antibodies and either left unstimulated or treated with 200 ng/ml TPA or 20 µg/ml forskolin. Fig. 3d[b]) The RKIP antisense vector, pAS-C143, downregulates expression of endogenous RKIP. NIH 3T3 cells were co-transfected with pAS-C143 and a GFP-expressing plasmid. GFP-positive cells were isolated by FACS and immunoblotted with indicated antibodies. Fig. 3e[c]) The activity of an

AP-1 reporter gene was measured in serum-starved or TPA-stimulated NIH 3T3 cells following co-transfection with RKIP antisense (pAS-C143) or empty vectors. --

Please replace the paragraph at page 24, from line 1 through line 13, with the following paragraph:

-- ~~FIG. 4 shows FIG. 4a – Fig. 4d show that RKIP inhibits Raf-induced AP-1 activation and transformation.~~ Fig. 4a) RKIP reduces basal and BXB-induced AP-1 activity in NIH 3T3 cells co-transfected with a 3xTRE-CAT reporter and the indicated expression plasmids. Fig. 4b) RKIP blocks BXB- but not ERK-induced AP-1 activation. Rat-1 cells were co-microinjected with a 4xTRE-lacZ reporter and the indicated expression vectors. Fig. 4c) RKIP inhibits Raf-dependent proliferation and transformation. NIH 3T3 cells were transfected with BXB, alone or together with RKIP (linked to neo). G418-resistant colonies were counted and scored for morphological transformation. Aliquots of the same transfection were allowed to grow to confluence without drug and were scored for focus formation. A BXB-transformed cell line was infected with LXSH-RKIP retrovirus or LXSH (hygromycin resistant) and seeded in soft agar in the presence of hygromycin. Fig. 4d) RKIP does not inhibit transformation by v-fos, v-src, or mutationally activated MEK in 208F or NIH cells. Data are expressed as reduction in focus formation relative to co-transfection with empty vector (set to 100%). --

Please replace the paragraph at page 24, from line 14 through line 22, with the following paragraph:

-- ~~FIG. 5 shows FIGs. 5a – Fig. 5e show that RKIP specifically blocks MEK phosphorylation by Raf-1.~~ Fig. 5a) Effect of RKIP on the activation steps of the Raf/MEK/ERK cascade reconstituted in vitro with purified recombinant proteins. "BSA" 15 uM bovine serum albumin; "Co." substrate alone; "kn", kinase negative mutant. Fig. 5b) RKIP does not inhibit activated MEK. HA-MEK-DD or HA-MEK-1 expressed in COS-1 cells were immunoprecipitated with anti-HA antibodies from serum starved cells or TPA treated cells, respectively, and assayed for kinase activity. Fig. 5c) RKIP does not inhibit MEK phosphorylation by MEKK-1. DELTA.MEKK-1 was immunoprecipitated from transiently transfected COS-1 cells and used to phosphorylate knMEK. Figs. 5d and Fig. 5e) RKIP does not inhibit Raf-1 autophosphorylation or phosphorylation of myelin basic protein (MBP). --

Please replace the paragraph at page 25, from line 1 through line 15, with the following paragraph:

-- FIG. 6 shows FIG. 6a – Fig. 6f show that RKIP regulates MEK and ERK activation in vivo. Fig. 6a) RKIP downregulation activates MEK. NIH 3T3 cells were co-transfected with GFP and the RKIP antisense plasmid, pAS-C143. GFP positive cells were FACS sorted and immunoblotted with the indicated antisera. Fig. 6b and Fig. 6c) RKIP antibody microinjection enhances ERK activation. Quiescent NIH 3T3 cells were microinjected with anti-RKIP or control IgG and stimulated with 10 ng/ml TPA for 30 minutes. ERK activation was visualized with a monoclonal anti-phospho-ERK antibody (Sigma) and quantified densitometrically. Fig. 6d[c]) RKIP inhibits MEK-1 activation. COS-1 cells were transiently transfected with HA-MEK and increasing amounts of RKIP expression vectors. Serum starved cells were stimulated with 100 ng/ml TPA for 20 minutes, and the kinase activities of RAF-1 and HA-MEK immunoprecipitates were measured. Fig. 6e[d]) RKIP inhibits stimulation of ERK by v-Ras and v-Src. COS-1 cells were transfected with the indicated expression plasmids plus increasing amounts of RKIP HA-ERK-2 was immunoprecipitated and assay with MBP. Fig. 6f[e]) RKIP inhibits ERK activation by BXB, but not by MEK-DD. COS-1 cells were transfected with the indicated expression vectors and the kinase activity of HA-ERK immunoprecipitates was examined. --

Please replace the paragraph at page 25, from line 16 through line 22 and page 26, from line 1 throuh 3, with the following paragraph:

-- FIG. 7 shows FIG. 7a – Fig. 7d shows that RKIP inhibits the ERK pathway by preventing MEK activation. Fig. 7a) Rat-cells were microinjected with a TRE-LacZ reporter plasmid and affinity-purified RKIP antibodies or preimmune immunoglobulin G (IgG) and treated as indicated. The MEK Inhibitors PD98059 and U0125 were administered 1 h before microinjection of TPA (110 ng/ml). Fig. 7b and Fig. 7c) RKIP antibodies prevent binding of RKIP to Raf-1 or MEK. GST, GST-RKIP, or GST-14-3-3 beads were incubated with saturating amounts of RKIP antibodies (I) or the corresponding preimmune serum (P) and tested for binding of Raf-1 or MEK 1. WB, Western blot. Fig. 7d[c]) The phosphorylation of kinase-

negative MEK-1 (knMEK) by activated Raf-1 was examined in the presence (+) or absence (-) of 10 μ M purified RKIP. RKIP was preincubated with RKIP antibodies or the corresponding preimmune serum for 1 h. --

Please replace the paragraph at page 26, from line 4 through line 18 with the following paragraph:

-- FIG. 8 shows FIG. 8a – Fig.8c show that RKIP inhibits Raf-1 by a competitive mechanism. Fig. 8a) Lineweaver-Burk plot of Raf-1 inhibition by RKIP. Activated GST-Raf-1 was used to phosphorylate GST-MEK-1 in the presence of increasing amounts of RKIP, as indicated. Phosphorylation was quantified with a Fuji phosphorimager. The data shown are the averages of three independent experiments. Fig. 8b) RKIP disrupts the Raf-1-MEK complexes. GST-MEK and Raf-1 were coexpressed in Sf-9 cells. The GST-MEK-Raf-1 complex was purified by adsorption to glutathione Sepharose beads, washed, and resuspended in PBS. Purified RKIP was added at the concentrations indicated. After 1 h at 4° C., the GST-MEK beads were washed three times with PBS and examined for associated proteins by Western blotting (WB) with the indicated antisera. Fig. 8c) Raf-1 bound to RKIP does not phosphorylate MEK. A lysate of Sf-9 cells expressing activated Raf-1 was incubated with 5 .mu.g of GST or GST-RKIP beads. Serial dilutions of the same lysate were immunoprecipitated with the anti-Raf serum crafVI. After three washes with PBS, the pellets were resuspended in kinase buffer and incubated with 100 μ M ATP and kinase-negative MEK as substrate. MEK phosphorylation was visualized by immunoblotting with a phospho-MEK-specific antiserum. Raf-1 was stained with crafVI. --

Please replace the paragraph at page 26, from line 19 through line 22, and page 27, line 1 through line10, with the following paragraph:

-- FIG. 9 shows FIG. 9a – Fig. 9d show an analysis of RKIP binding to activated Raf-1, MEK, and ERK. Fig. 9a) Mitogen activation of Raf-1 decreases its association with RKIP. COS-1 cells were transiently transfected with Raf-1 and RKIP expression vectors. Serum-starved cells were treated with epidermal growth factor (EGF) (20 ng/ml) plus TPA (100 ng/ml) for the times indicated. Raf-1 immunoprecipitates were analyzed for kinase activity, and RKIP immunoprecipitates were examined for Raf-1, IP, Immunoprecipitation, WB, Western blot. Fig. 9b) Purified RKIP produced in E. coli was tested for binding to GST-Raf and activated GST-Raf

beads. GST-Raf proteins were produced in Sf-9 cells and activated by coexpression of RasV12 and Lck. An aliquot of the GST-Raf beads was examined for phosphorylation of kinase-negative MEK (knMEK). (Fig. 9c and Fig. 9d) MEK and ERK proteins were phosphorylated in the presence of [γ -³²P]ATP and tested for binding to GST-RKIP beads. Binding of phosphorylated proteins was detected by autoradiography. Binding of total protein was visualized by Western blotting (WB). The contribution of phosphoproteins to the Western blot signal is minimal, because they represent less than 10% of the total protein. --

Please replace the paragraph at page 27, from line 11 through line 15, with the following paragraph:

-- ~~FIG. 10 shows FIG. 10a and Fig. 10b show~~ that RKIP binding of Raf-1 decreases during mitogenic stimulation. Serum starved rat-1 cells were treated with 20% fetal calf serum for the indicated timepoints. Fig. 10a) Raf-1 immunoprecipitates were immunoblotted for associated RKIP. Fig. 10b) Cell lysates were examined for RKIP and ERK expression. ERK activation was monitored with a phospho-ERK specific antibody. --

Please replace the paragraph at page 27, from line 16 through line 22, and page 28, line 1 through line 3, with the following paragraph:

-- ~~FIG. 11 shows FIG. 11a – Fig. 11g show~~ an analysis of binding domains. Fig. 11a and Fig. 11 b) RKIP and MEK bind to different domains of the Raf-1 kinase. GST-tagged BXB, GNX, and the indicated deletion mutants were expressed in E. coli, immobilized on glutathione Sepharose beads, and incubated with purified RKIP or MEK-1. Proteins were visualized by Western blotting. The diagram illustrates the GNX regions deduced to be required for binding. Roman numerals refer to the kinase subdomains as defined by Hanks and Quinn. Fig. 11c, Fig. 11d and Fig. 11e[b]] RKIP and Raf-1 bind to different domains of MEK-1. Purified six-His-tagged MEK-1 deletion mutants were tested for binding to GST-RKIP beads (Fig. 11c right panel) and GST-Raf-1 beads (Fig. 11d left panel). His/MEK-1 proteins were detected by Western blotting with anti-His antibodies. Fig. 11e ~~The lower panel~~ shows a schematic summary.

nd, not done. Fig. 11f and Fig. 11g[c]) Analysis of Raf-1 and MEK binding sites in RKIP, GST-RKIP deletion mutants were tested for binding of MEK-1 and Raf-1. PEB, phosphatidylethanolamine binding motif. --

Please replace the paragraph at page 28, from line 4 through line 9, with the following paragraph:

-- ~~FIG. 12 shows FIG. 12a – Fig 12d~~ show an analysis of the composition of RKIP protein complexes. Fig. 12a) GST-MEK beads were incubated with RKIP, Raf, and MEK in the indicated combinations. GST-RKIP beads Fig. 12b), GST-ERK beads Fig. 12c), or GST-Raf-1 beads Fig. 12d) were incubated with recombinant purified proteins as indicated. Incubations were done as described in Materials and Methods, and associated proteins were visualized by Western blotting. --

Please replace the paragraph at page 28, from line 10 through line 17, with the following paragraph:

-- ~~FIG. 13 shows FIG. 13a – Fig. 13c~~ show that RKIP binding to Raf-1 or MEK is sufficient for inhibition. Fig. 13a and Fig. 13b) Coimmunoprecipitation of RKIP deletion mutants with Raf-1. FLAG-Raf-1 and hemagglurinin (HA)-RKIP or HA-RKIP deletion mutants were coexpressed in COS cells. Lysates were immunoprecipitated (IP) with anti-FLAG antibodies, and associated HA-RKIP proteins were detected by Western blotting (WB) with anti-HA antibodies. PEB, phosphatidylethanolamine binding motif. Fig. 13c[b]) The effect of RKIP deletion mutants on Raf-induced AP-1 reporter gene expression. HA-RKIP mutants were cotransfected with the Raf-1 kinase domain, BXB, and an AP-1-luciferase plasmid. --